

EXHIBIT D

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Dopamine D2 receptor dimers in human and rat brain

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Abstract In order to determine whether dimers of dopamine D2 receptors can occur in mammalian brain, rat and human brain striatal membranes were photolabelled with two radioactive photoaffinity compounds selective for dopamine D2 receptors, [¹²⁵I]azidophenethylspiperone and [¹²⁵I]-4-azido-5-iodonemonapride. It was found that [¹²⁵I]azidophenethylspiperone only labelled the D2 monomer, while [¹²⁵I]-4-azido-5-iodonemonapride labelled both D2 monomers and dimers, despite the fact that very high concentrations (6 nM) of both radiocompounds were used. In addition, human cloned D2 receptors were probed with a D2-specific antibody, revealing multiple bands indicating the existence of trimers, tetramers and pentamers of D2 receptors. The different D2-binding patterns of the spiperone and benzamide congeners may explain the different densities of dopamine D2 receptors found with these two radioligands in human brain positron tomography in health and disease.

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Key words: Dopamine receptor; Photoaffinity labelling; Receptor dimer

1. Introduction

In measuring brain neurotransmitter receptors in patients by means of positron tomography, a long-standing problem has been the fact that two different radioligands yield significantly different densities for the same receptor. The problem is further compounded when the receptor is measured in disease. For example, the density of brain dopamine D2 receptors in healthy volunteers is 15–16.7 pmol/ml when measured with [¹²⁵I]methylspiperone [1,2], but, even in the same laboratory [3], is 25 pmol/ml when measured with [¹²⁵I]raclopride. Farde et al. have also found a difference in these radioligand densities in healthy subjects [4,5] and in schizophrenia patients [5,6]. An analogous situation occurs *in vitro* in a variety of tissues, including cloned dopamine receptors [7–10]. Such discrepancy revealed by the two radioligands may be clinically important in studying the dopamine hypothesis of schizophrenia, because one research team finds D2 receptors to be elevated in schizophrenia when using [¹²⁵I]methylspiperone [1,2], while another research team finds no elevation in D2 receptors when using [¹²⁵I]raclopride [4,6]. The discrepancy suggests that the two radioligands may bind differently to the D2 receptor. We here offer a partial explanation by showing that D2 receptors can exist either as monomers or dimers in mammalian brain tissue, with radioisotope labelling the mono-

mer, while radionemonapride labels both forms of D2, a situation similar to that for cloned D2 receptors which we have reported previously to exist in monomer and dimer forms [11–13].

2. Materials and methods

2.1. Preparation of rat striatum tissue

Frozen rat brains were purchased (Pel-Freez Biologicals, Rogers, AR, USA), thawed, and the striata removed. A total of 18 pmol of D2 receptors was prepared from 25 frozen rat brains. (The D2 receptor density in rat striatum is ~30 pmol per gram of original striatum [7,8].) The tissue was suspended in 1 ml of 0.25 M sucrose in buffer (5 mM Tris-HCl, pH 7.6, 0.5 mM MgCl₂, and 2 µl of protease inhibitor mixture (Boehringer Mannheim, Laval, Que., Canada) per ml of buffer), and homogenized in 10 up-and-down strokes, using a Teflon-glass hand homogenizer (2 ml). A crude enrichment of striatal synaptosomes was prepared as follows. The homogenate was centrifuged for 5 min at 280 × g. The supernatant was saved, while the pellet was resuspended in 1 ml of 0.25 M sucrose in buffer and re-centrifuged for 5 min at 280 × g. The first and second supernatants were combined and centrifuged for 10 min at 1500 × g. The resulting pellet was resuspended in 1.46 M sucrose in buffer. Two aliquots of 30 ml each were placed in 40-ml Beckman polyallomer centrifuge tubes and overlaid with 7 ml of 0.25 M sucrose in buffer. After centrifugation at 24000 × g for 60 min, the material at the interface of the sucrose gradient was collected, using a blunt-tipped Pasteur pipette, and resuspended in 0.43 M sucrose in buffer. The suspension was centrifuged at 1500 × g for 10 min. The final pellet was resuspended in 0.25 M sucrose in buffer. All procedures were done at 4°C.

2.2. Preparation of post-mortem human brain caudate nucleus tissue

Frozen samples of post-mortem human brain caudate nucleus were obtained from the Canadian Brain Tissue Bank (CBTB). The tissues provided were from patients CBTB 355 and CBTB 1271, both of whom had Alzheimer's disease. Patient CBTB 355 developed Alzheimer's disease at age 54 and progressively deteriorated over the next ten years, receiving only phenytoin and ampicillin in the months before dying at age 64. The post-mortem death-to-freezing interval was 10 h. Patient CBTB 1271 developed Alzheimer's disease at age 56, but also revealed metastatic adenocarcinoma over the next 6 months. He died at age 57, having taken anti-cancer medication in the months before death. The post-mortem death-to-freezing interval was 5.5 h. The frozen tissue samples were homogenized by means of sonication (3 min; Branson) in buffer (5 mM Tris-HCl, pH 7.4, 2 mM EDTA, with protease inhibitors as outlined above). The homogenate was centrifuged at 500 × g for 10 min. The supernatant was centrifuged at 50000 × g for 20 min, resuspended in buffer and re-centrifuged at 50000 × g for 20 min.

2.3. Photoaffinity compounds for labelling dopamine D2 receptors

Two photoaffinity compounds were used to label dopamine D2 receptors: [¹²⁵I]-4-azido-5-iodonemonapride ([¹²⁵I]-A-YM) and N-[4-azido-5-iodophenethyl]spiperone ([¹²⁵I]-A-PS). N-[4-azido-5-iodophenethyl]spiperone has previously been used to photolabel dopamine D2 receptors [14].

[¹²⁵I]-4-Azido-5-iodonemonapride was prepared as follows. The amino compound, 4-normethyl-5-deschloronemonapride (Research

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Biochemical International MH-Y-705), was synthesized and provided by Drs. R.A. Milius and J.L. Neumeyer (Research Biochemical International, Natick, MA, USA). (Nemopamine), (Nemopamine), formerly known as YM-09151-2, is (+)-*cis*-N-(1-benzyl-2-methyl-pyrrolidin-3-yl)-5-chloro-2-methoxy-4-methylaminobenzenamide [7,15,16]. Thereafter, 4-normethyl-5-deschloronemopamine (RBI MH-Y-705) was converted to either the non-radioactive azido compound, 4-azido-5-iodonemopamine (or RBI MH-Y-704), or to the radioactive photolabel azido compound, [¹²⁵I]-4-azido-5-iodonemopamine (known as [¹²⁵I]azido-MH-Y-705, catalog No. NEX-084 or NET-999D00MOC, lot No. 2903214, custom-prepared by New England Nuclear Life Science Products, DuPont de Nemours), by means of iodination with chloramine-T.

Both N-(p-azido-methyl)iodophenethylpiperone and [¹²⁵I]-4-azido-5-iodonemopamine were prepared carrier-free, each with a specific activity of 2200 Ci/mole. Because these radioactive molecules self-destruct upon the release of the gamma particle, the molarity decreased with time, but the specific activity remained constant.

2.4. Photolabeling of D2 receptors expressed in Sf9 cells

Human recombinant dopamine receptors, type 2_{long}, purchased from Biogal (Montreal, Que., Canada), had been overexpressed in baculovirus-infected *Spodoptera frugiperda* Sf9 insect cells (cultured in Grace's medium supplemented with 10% fetal bovine serum). Aliquots of Biogal membranes (25–100 µg protein) were centrifuged at 14000 rpm for 5 min at 4°C (centrifuge 541SC, Brinkmann Instruments, Toronto, Canada). The membrane pellets were washed twice in 50 mM Tris-HCl, pH 7.5, and finally resuspended in 50 mM Tris-HCl (pH 7.5) at protein concentrations of 3–12 µg/µl. To minimize protein degradation, protease inhibitor cocktail tablets were added according to Boehringer Mannheim's specifications.

To label the receptors, the final concentrations of the radiophotolabels were between 0.3 and 20 nM in a final volume of 35 µl, containing 50 µg of rat brain striatal membrane protein. Non-specific binding was that which occurred in the presence of 100 nM (+)-butaclamol or 10 µM 5-sulpiride. Protease inhibitor cocktail tablets were also added according to Boehringer Mannheim's specifications. The membranes were incubated in the dark with either 2–6 nM [¹²⁵I]-A-YM or 2–4 nM [¹²⁵I]NAPS at room temperature (22°C) in a final volume of 35 µl, using a 1-ml plastic conical centrifuge tube. The samples were then placed on ice, and the tops of the tubes were exposed to ultraviolet light (365 nm) for 1.5 min. The tubes were centrifuged at 14000 rpm for 8 min at 4°C (centrifuge 541SC, Brinkmann Instruments). The surfaces of the pellets were rinsed twice with 50 mM Tris-HCl (pH 7.5), and each pellet finally solubilized (one part pellet to two parts SDS buffer) in 35 µl of SDS buffer (50 mM Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue) and analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The samples were loaded onto 10-well, 1.5-mm, 8–16% Tris-glycine gels, and run for approximately 2 h at 135 V. Each gel was then incubated with gentle mixing in 50 ml of methanol/acetic acid (40:18%) for 1.5 h at 22°C. The gels were dried under vacuum for 1.5 h at 80°C using a Bio-Rad model 583 gel dryer. The gels were overlaid with Kodak Scientific Imaging Film, placed in double-sided high-intensity screen autoradiography cassettes, and stored at –70°C for 1–60 day exposures. Films were developed using a Kodak model M35A X-OMAT diagnostic imaging film processor. The molecular mass of proteins was determined using apparent molecular weights from the pre-stained protein marker SuperBlue 2360.

2.5. Immunoblot analysis

After completing the SDS-PAGE procedure, the samples were transferred onto a nitrocellulose membrane (0.2 µm), using 30 V for 2.5 h. The nitrocellulose membrane was blocked for 1 h with 5% skim milk powder and 1.5% bovine serum albumin in Tween 20/Tris-buffered saline (TTBS: 0.1% Tween 20, 100 mM Tris-HCl, pH 7.5, and 0.9% NaCl). Once blocked, the nitrocellulose membrane was washed for 1 h in Tris-buffered saline (100 mM Tris-HCl, pH 7.5, and 0.9% NaCl) and then incubated overnight at 4°C with the AL-26 polyclonal primary antibody dissolved in TBS (1:1000 dilution). This antibody is directed against a 120-amino acid sequence (N-terminus 66–102) in the third intracellular loop of the human D₂ receptor [17]. The immunoblot was then washed for 1 h in TTBS, followed by incubation for 3 h at 22°C with a secondary antibody, protein [¹²⁵I]-protein-A (final concentration of 1 µCi/ml; New England Nuclear Life Science Products, DuPont de Nemours, Boston, MA,

USA), dissolved in TBS (1:5000 dilution). The immunoblotted nitrocellulose membrane was then exposed to Kodak Imaging film using double-sided high-intensity screens for 1–3 days at –70°C and developed as described above.

3. Results

Because earlier work on bovine and pig tissues had not detected D2 dimers [18], while later work on cloned and highly expressed D2 receptors did detect dimers [11–13], we thought it essential to enrich the D2 receptors in result to detect D2 dimers in rat brain striatum. The result is shown in Fig. 1, where 6 nM [¹²⁵I]azidophenethylpiperone detected only the glycosylated monomer of D2 at 120 kDa (lane 1), while [¹²⁵I]-4-azido-5-iodonemopamine detected both the monomer and the dimer (~250 kDa) of the D2 receptor (lane 3). (The high concentration of 6 nM was chosen to ensure that most of the receptors would be occupied by each radiophotolabel, because the dissociation constant of 4-azido-5-iodonemopamine (RBI MH-Y-704) was 0.46 nM on human cloned dopamine D2 receptors, using 250 pM [³H]piperone to label these receptors (unpublished), and because the dissociation constant of [¹²⁵I]azidophenethylpiperone was also of the same order of magnitude [12] as 4-azido-5-iodonemopamine.)

The results in Fig. 1 for rat brain striatum are similar to those for human cloned D2 receptors expressed in insect Sf9 cells, as shown in Fig. 2. This Fig. 2 contains additional control data, indicating that the photolabeling by [¹²⁵I]-4-azido-5-iodonemopamine was stereoselectively blocked by (+)-butaclamol and not by (–)-butaclamol (lanes 2 and 3), and that the Sf9 cells alone (i.e. not expressing D2 receptors) were not photolabelled in the regions of 125 and 250 kDa (lanes 6 and 7).

Fig. 3 illustrates two examples of results on post-mortem human caudate nucleus tissue from individuals who had Alzheimer's disease. The example on the left in Fig. 3 shows that [¹²⁵I]azidophenethylpiperone primarily labelled the D2 monomer in brain CBTB 1271, and the labelling was occluded by 4 µM (+)-butaclamol. In the example shown on the right in

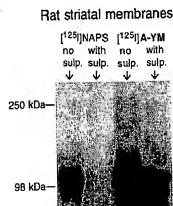


Fig. 1. Dopamine D2 receptors in rat brain striatal membranes were photolabelled with 6 nM [¹²⁵I]azidophenethylpiperone ([¹²⁵I]NAPS) or 6 nM [¹²⁵I]-4-azido-5-iodonemopamine ([¹²⁵I]A-YM), subjected to SDS-PAGE, and exposed to Kodak imaging film. The glycosylated monomer of D2, with an MW of ~120 kDa, was photolabelled by [¹²⁵I]NAPS (lane 1) and by [¹²⁵I]A-YM (lane 3). However, only [¹²⁵I]A-YM labelled the D2 dimer, with an MW of ~250 kDa, as shown in lane 3. Non-specific binding was that which occurred in the presence of 10 µM 5-sulpiride.

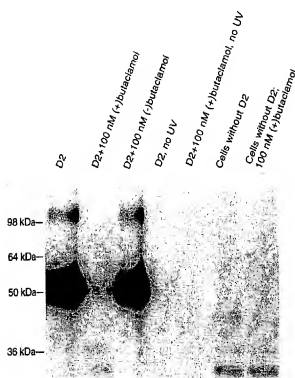


Fig. 2. Human cloned dopamine D2 receptors photolabelled with 6 nM [125 I]-4-azido-5-iodonemopride ([125 I]-A-YM), subjected to SDS-PAGE, and exposed to Kodak imaging film. Non-specific binding was defined as that occurring in the presence of 100 nM (+)-butaclamol.

Fig. 3 for brain CBTB 355, [125 I]-4-azido-5-iodonemopride primarily labelled D2 dimers. We were not able to determine what biochemical factors or variables in the procedure would lead to a preponderance of dimers for either human or rat brain striatal tissues.

In order to obtain evidence for the existence of D2 dimers by means of another method, human cloned D2 receptors,

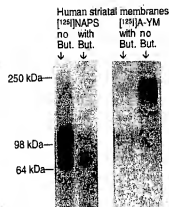


Fig. 3. Dopamine D2 receptors in post-mortem human brain caudate nucleus membranes were photolabelled with 10 nM [125 I]-4-azido-5-iodonemopride ([125 I]-A-YM) or 20 nM [125 I]-azidophenethyl-spiperone ([125 I]-NA-PS), subjected to SDS-PAGE, and exposed to Kodak imaging film. Left: Brain CBTB 1271 (Alzheimer's disease): the glycosylated monomer of D2, with an MW of ~ 100 kDa, was photolabelled by [125 I]-NA-PS with a faint band appearing at ~ 200 kDa (lane 1), both bands of which did not appear in the presence of 4 μ M (+)-butaclamol (lane 2). Right: Brain CBTB 355 (Alzheimer's disease): although the monomer of D2, with an MW of ~ 100 kDa, did not appear here with [125 I]-A-YM, the dimer band of ~ 200 kDa (lane 3) did appear, this band not showing in the presence of 4 μ M (+)-butaclamol (lane 4).



Fig. 4. Human cloned dopamine D2 receptors immunoblotted with D2 antibody and [125 I]protein-A. D2 receptors (expressed in insect Sf9 cells) were subjected to SDS-PAGE (25 μ g protein per lane), immunoblotted with a D2-specific antibody, and detected with [125 I]protein-A (1 μ Ci/ml). Control samples, containing wild-type Sf9 cells (transfected with baculovirus, but not expressing D2 receptors) did not show any immunoreactive bands.

expressed in insect *Spodoptera frugiperda* cells, were immunoblotted and probed with the D2-specific antibody AL-26, the results of which are shown in Fig. 4. This figure reveals bands for [125 I]protein-A at ~ 55 kDa, ~ 110 kDa, ~ 158 kDa, ~ 221 kDa, and ~ 347 kDa, suggesting the existence of not only monomers and dimers of D2, but also trimers, tetramers and pentamers of D2, compatible with the concept of higher order oligomers for seven-transmembrane-G-linked receptors [19,20].

4. Discussion

Figs. 1 and 2 indicate that D2 dimers can occur in rat brain striatal membranes as well as in human cloned D2 receptors. As monitored by photoaffinity labelling, the proportion of dimers is at least 1–5% of the monomer fraction, as indicated by the relative intensities shown in Figs. 1 and 2. The proportion may be much higher than 1–5%, because the photoaffinity labelling procedure may result in a low total number of receptors to become labelled.

The immunoblot data (Fig. 4) provided more evidence for the existence of D2 dimers, while also indicating the existence of trimers, tetramers and pentamers of D2. The [125 I]protein-A bands in Fig. 4 correspond to the appropriate molecular weights of D2 monomers (~ 48 kDa) and dimers (~ 98 kDa), as predicted by the receptor's amino acid sequence.

Many signal transducing receptors, including growth factor receptors and cytokine receptors, depend on the formation of dimers for proper function. For example, disruption of dimers of the β -adrenoceptor inhibits its function [21]. Receptor dimers may also regulate desensitization, endocytosis, and recycling of receptors. Agonist exposure may increase or decrease the dimer:monomer ratio [21,22], while inverse agonists decrease this ratio [21], suggesting that interconversion between monomers and dimers may be important for biological activity. It will be essential to examine these physiological processes for D2 receptors in health and disease. For example, it is possible that there may be a monomer-dimer imbalance in dopaminergic diseases such as schizophrenia.

The data in Fig. 4 suggest the existence of additional higher order oligomers, namely, trimers, tetramers and pentamers. However, since protein-A can also bind non-specifically to IgG molecules, it is also possible that these bands may repre-

sent other proteins expressed within the cell but which are not related to dopamine D2 receptors.

An important observation in Fig. 1 is that [125 I]azidophenethylspiperone only labelled the rat brain D2 monomer, while [125 I]-4-azido-5-iodonemonaipride labelled both the D2 monomers and dimers, despite the fact that very high concentrations (6 nM) of both radiocompounds were used. These data suggest that the spiperone and benzamide congeners may label different populations of the D2 receptor, possibly resolving the density discrepancies found with these two radioligands in positron tomography [1-6]. In other words, because the benzamide attaches to both monomers and dimers, the density of [14 C]raclopride sites would be expected to exceed the density of D2 monomer sites labelled by [14 C]methylspiperone, as is the case (see Section 1).

The apparent selectivity of [125 I]azidophenethylspiperone to detect monomers of D2 but not dimers or higher order oligomers may be attributed to 'negative cooperativity'. This mechanism of negative cooperativity as the basis for multiple states of receptor affinity has been previously applied to the cardiac muscarinic receptor [19,23]. These latter studies suggested that negative cooperativity may explain differences in the relative receptor densities observed with two different muscarinic radioligands. Hence, receptor oligomers may also explain why different radioligands yield different densities for the G protein-coupled D2 receptor [8]. Moreover, negative cooperativity between receptors in oligomeric species may result in decreased affinity towards specific ligands.

Although the D2 antibody was capable of detecting higher order oligomers of D2, the [125 I]-4-azido-5-iodonemonaipride was not able to detect them. It is possible, therefore, that the structural conformation of the D2 receptor in these oligomeric states may make the binding pocket inaccessible to the photolabel, while not interfering with the binding sites for the antibody.

The present experiments observed D2 receptor monomers, dimers, and higher order oligomers by means of SDS-PAGE. The results only reveal receptor species that are resistant to the detergent SDS. In the absence of disulfide bonds, the oligomeric forms of the receptor must be held together by strong inter- and intramolecular forces, as predicted by Maggio et al. [24]. However, the low proportion of dimers, compared to the abundance of monomers in the photolabel experiments (but not in the [125 I]protein-A data) suggests that the SDS-PAGE method may disrupt the dimer form of the receptor.

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